

Activity Testing of Alveolar Macrophages and Changes in Surfactant Phospholipids after Irradiation in Bronchoalveolar Lavage: Experimental and Clinical Data

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This study presents results of bronchoalveolar lavage (BAL) after irradiation to the lungs in mice as well as clinical data. The number of BAL cells, mainly macrophages, lymphocytes, and granulocytes, changed in a time-dependent manner. The phagocytic activity of the macrophages measured as the phagocytosis of microbeads and measured as the esterase activity also showed a strong time-dependent increase during the acute phase up to 21 days after irradiation. The contents of surfactant phospholipids (SF) and sphingomyelin (SPH; as a parameter for cell death) were quantified by HPLC. Both were significantly changed between day 2 and 21 after irradiation. Three BALs of a patient with idiopathic interstitial pneumonitis, who had received an allogeneic bone marrow graft after total body irradiation with 10 Gy, showed similar effects in the cellular and surfactant parameters. These data indicate that there are positive interactions between the number of different BAL cells, macrophage activity, and SF and SPH content in the preclinical model of the mouse as well as in the clinical situation after lung irradiation.

Introduction

In thoracic and total body irradiation (TBI), the lung represents a dose-limiting organ. In the setting of bone marrow transplantation, TBI provides a high immunosuppressive and antitumor effect. However, a number of patients may develop idiopathic interstitial pneumonitis (IIP), which may partially be induced by radiation injury. The clinical course of IIP is identical to the adult respiratory distress syndrome (ARDS) in polytrauma patients, which can develop dramatically and attain a stage that can no longer be controlled by therapy. The underlying pathogenic mechanism is still unknown.

The epithelial lining fluid (ELF) of the respiratory tract may serve as an indicator of pulmonary injury or disease. ELF cannot be obtained as easily as blood or urine, but bronchoalveolar lavage (BAL) with physiological saline can be used to sample ELF in animal experiments and in clinical situations. Recently, the analysis of BAL has proved useful for detecting an in-

flammatory response in the lungs of animals exposed in toxicology studies, and there is optimism about the use of BAL analysis as an early predictor of late-occurring pulmonary disease (*1*). BAL can be used as an aid in studying the pathogenic mechanism of diseases such as ARDS or IIP. However, these techniques have not generally been used in association with TBI. Up to now, only few largely experimental animal data are available on the reaction mechanism of surfactant-alveolar-macrophage-P II (pneumocyte type II) cells. This study presents results of BAL after single irradiation (10 Gy) to the lungs in mice with measurements of the total cell count (granulocytes, macrophages, and lymphocytes), assays of macrophage esterase activity and the activity of macrophages after application of microbeads, and quantification of the sphingomyelin content as an important surfactant phospholipid fraction in the BAL indicative of cell death. Because pulmonary complications are a major cause of morbidity and mortality in patients (2-4) undergoing bone marrow transplantation with a preconditioning whole body irradiation (5,6), the above-mentioned parameters from a patient with IIP after bone marrow transplantation will be demonstrated and discussed.

Materials and Methods

Radiation. A single dose of 10 Gy (1 Gy/min) was applied to the thorax of Heiligenberger mice (male, weight 28-35 g) using

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an X-ray machine (Stabilipan, Siemens, Erlangen, FRG) operating at 240 kV and 15 mA with a copper filter of 0.5 mm thickness. The field size was 18×16 cm and the focus skin distance was 47 cm. The animals were given 5 μ g/g body weight penobarbital intraperitoneally for anesthesia.

Cell Differentiation. We sacrificed the animals by intraperitoneal injection of barbiturate 2, 7, 14, and 21 days after irradiation. The lung was dissected free and the trachea cannulated with a syringe needle. By means of a syringe, the lung was washed with sterile, ice-cold physiological saline (10 times with aliquots of 0.8 mL). The BAL fluid was centrifuged at 180g for 10 min. We washed the cells with phosphate-buffered saline (PBS Dulbecco without Ca^{2+} and Mg^{2+} , pH 7.4; Seromed, FRG) and resuspended in incubation medium and counted in a Coulter counter (Coulter Electronics, Harpenden, UK). Macrophages, lymphocytes, and granulocytes were counted. Cell viability after washing was greater than 95% as determined by trypan blue dye exclusion.

Macrophage Activity. A total of $0.5\text{--}1 \times 10^6$ alveolar macrophages (2-mL cell suspension) was preincubated in a moist atmosphere containing 5% CO_2 (plastic petri dishes, 35 mm in diameter). Fluorescent and carboxylated latex beads (2 μ m in diameter) were added 1 hr later. The particle to cell ratio was 20:1. After a 2-hr incubation, we fixed the cells and examined them under a microscope. Phagocytic activity was defined as the percentage of cells that have shown phagocytosis. For details see Rehn et al. (7).

For assays of esterase activity, cells were smeared on a glass slide and treated with an unspecific esterase stain (α -naphthyl acetate, *p*-rosaniline) according to Davies and Ornstein (8) and embedded. By means of the previously described Image Analysing System Quantimet 970 (Cambridge, UK) (9), the optical density of each individual cell was measured using a bright light microscope. With the help of grey value calibration in the measuring system, 64 different channels could be detected. A distribution histogram was prepared from the mean values of all cells using the SAS software (SAS Institute Inc., Cary, NC).

Quantification of Sphingomyelin. Phospholipids were measured after separation by high pressure liquid chromatography (HPLC) (Krontron, Munich, FRG) at a constant temperature of 30°C and flow rate of 1 mL/min using a Biosil HP10, 10 μ m column (250 \times 4 mm i.d.) and a precolumn of Bio-Sil HP-10, 40 \times 4.6 mm (BioRad, Munich, FRG) at 202–210 nm with an Uvikon detector (Kontron, Munich, FRG) (10). The total phospholipid content and sphingomyelin (SPH) were extracted by the method of Folch et al. (11) and pooled for each group. For reference studies and calibration, the phospholipids were purchased from Sigma (Munich, FRG).

Case Report. A 43-year-old male with chronic myeloid leukemia received an allogeneic marrow graft from his HLA-identical brother after receiving fractionated whole body irradiation of 4×2.5 Gy (lung dose: 4×2.0 Gy) and 120 mg/kg body weight cyclophosphamide for conditioning. One hundred seven days after transplantation he developed an IIP with a distinct central cyanosis (Po_2 54 mm Hg, Pco_2 32.5 mm Hg, pH 7.44). To obtain a diagnosis of the underlying lung disease, BAL was performed under local anesthesia with a fiberoptic bronchoscope in a segment of the middle lobe with 6×20 mL of sterile saline solution and recovered by manual syringe suction [for details see

Leskinen et al. (12)]. The BAL fluid and the cells were then examined as described above.

Statistical Evaluation. Calculations were carried out using the SAS system (SAS, version 6; SAS Institute Inc., Cary, NC). Unless otherwise stated, results were statistically significant at $p < 0.05$.

Results

BAL Cell Count. Two days after irradiation, the total cell count was significantly lower than in control group (Fig. 1). It is of special interest that the first value (2 days after radiation) was already significantly below that of the controls. During the course of time, the value rose to the same level as in the controls. A similar effect is demonstrated for the number of macrophages where the values never rose above those of the control group (Fig. 2). Figures 3 and 4 show the time course of events for the number of granulocytes and lymphocytes in the lung after irradiation. In both cases, the day 21 value shows a maximum, significantly higher value than the controls.

Macrophage Activity. The phagocytic activity of the BAL macrophages, measured as the number of cells with phagocytized beads, showed a significant decrease in the percentage of cells on day 21 after irradiation (Fig 5). Similarly, the esterase activity in the 64 measured channels of optical density showed a comparable effect on day 21 (Fig. 6).

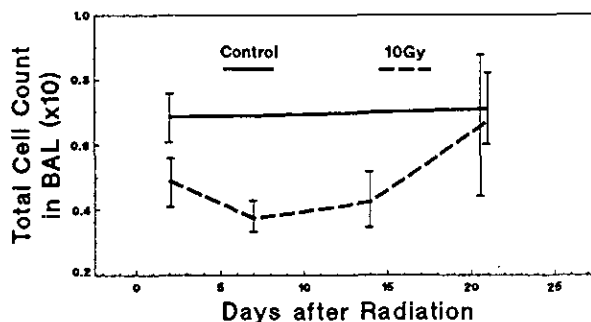


FIGURE 1. Total cell count in bronchoalveolar lavage of irradiated lung (10 Gy) and control mice. Values show means and SE ($n = 8$).

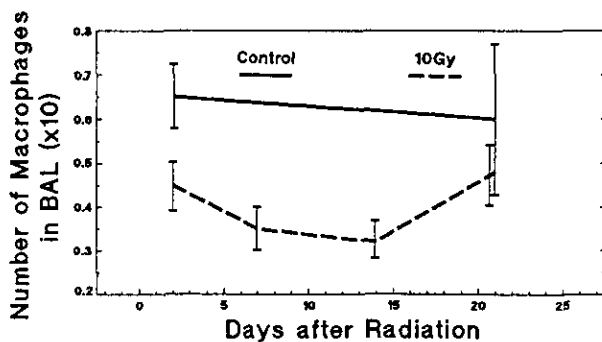


FIGURE 2. Number of macrophages in bronchoalveolar lavage of irradiated lung (10 Gy) and control mice. Values show means and SE ($n = 8$).

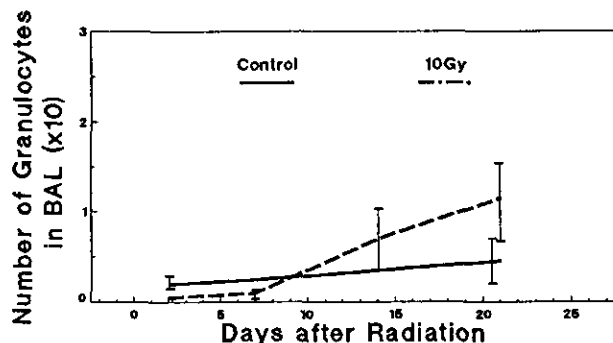


FIGURE 3. Number of granulocytes in bronchoalveolar lavage of irradiated lung (10 Gy) and control mice. Values show means and SE ($n = 8$).

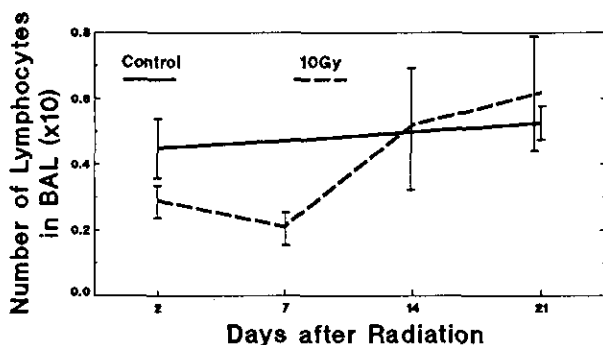


FIGURE 4. Number of lymphocytes in bronchoalveolar lavage of irradiated lung (10 Gy) and control mice. Values show means and SE ($n = 8$).

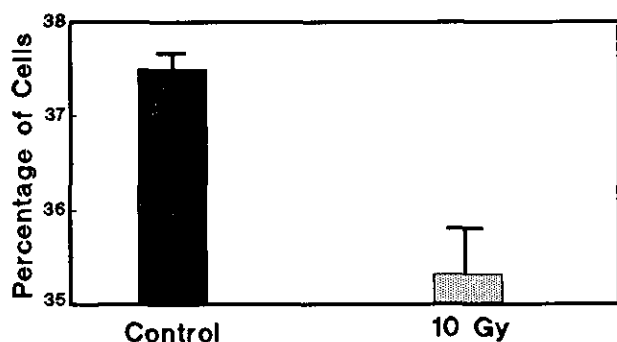
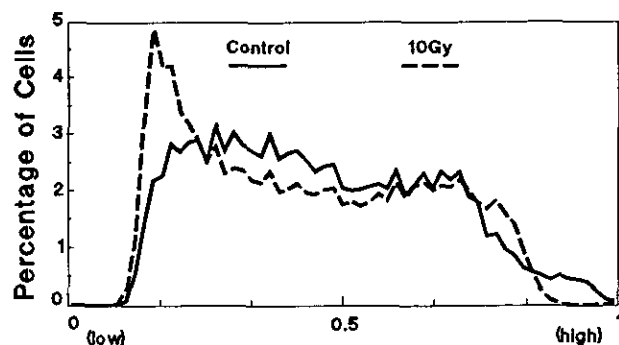


FIGURE 5. Percentage of phagocytizing macrophages in bronchoalveolar lavage of irradiated lung (10 Gy) and control mice. Values show means and SE ($n = 8$); significance at $p < 0.05$.

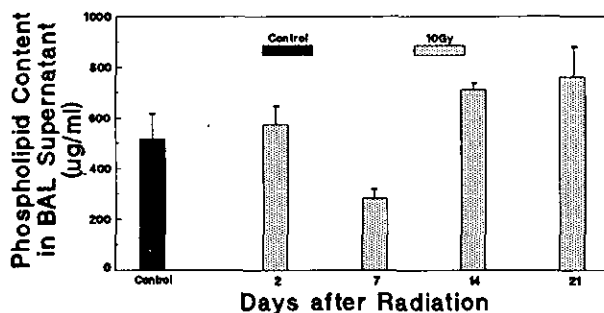
Phospholipid Surfactant and Sphingomyelin. An initial depression in the total phospholipid contents seen on day 7, after which the values rose again significantly above control levels (Fig. 7A). Sphingomyelin, which is an indicator for cell death, increased in parallel to the development of granulocytes and lymphocytes (Fig. 7B).

Patient Data. After the appearance of a clinically diagnosed IIP, two consecutive BALs were carried out on day 107 and 109 after transplantation. There were no signs of bacterial, viral, or fungal infections, but a significant rise of lymphocytes and granulocytes in differential cytology. On the basis of these findings, no antibacterial, antiviral or antifungal medication was taken.

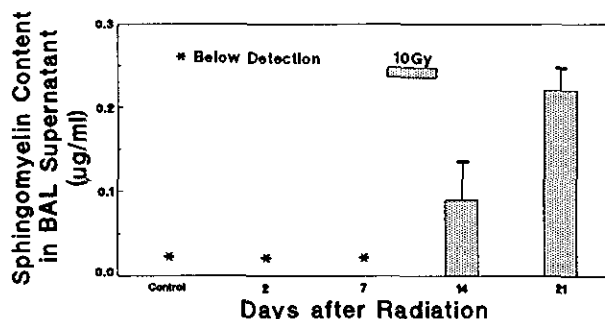


Measured by Optical Transmission

FIGURE 6. Shift in distribution of macrophages classified by their esterase activity at 21 days after radiation. The distributions are mean values of eight animals.



A



B

FIGURE 7. (A) Total surfactant phospholipid content and (B) sphingomyelin in bronchoalveolar supernatant of irradiated lung (10 Gy) and control mice. Values show means and SE ($n = 8$).

Instead, prednisone was administered IV at a dose of 75 mg/kg body weight daily and from day 4 onward given *per os* at a dose of 40 mg/kg daily, which after 3 days led to a PO_2 of 67 mm Hg and after 15 days to a PO_2 of 80 mm Hg. The radiological signs of interstitial pneumonitis cleared completely after 4 weeks of prednisone therapy. A control BAL was carried out on day 145 after transplantation (= 35 days after start of the therapy). Table 1 shows that the total cell count on day 107 after transplantation was relatively low. In particular, the low number of macrophages as well as the numerous lymphocytes and granulocytes are indicative of an acute process with ensuing immunodepression. On day 145 after transplantation, the decrease in total cell count

On day 145 after transplantation, the decrease in total cell count and the reduced number of granulocytes with a concomitant increase in macrophages indicate that the acute phase is beginning to decline. The high levels of SPH (Table 2) on day 109 point to a massive destruction of cells. On day 145, the percentage of macrophages clearly rose, while that of the SPH was apparently lowered; the healing phase had not set in, as made evident by these parameters. Table 3 shows the activity of the macrophages. Here, in contrast, no significant changes in the macrophage activity can be seen. The BAL phospholipid content increases more than 11-fold up to day 145 after transplantation. The amount of SPH, which was still below the level of detection on the first day of BAL, reached a maximum in parallel to the highest number of macrophages (Table 2) and total cell counts (Table 1).

Discussion

The lung has been shown to be a dose-limiting organ both for TBI in bone marrow transplantation and after partial-body irradiation of the thorax (13-16). A review by Hamilton et al. (17) of clinical reports on damage to the lung after bone marrow transplantation lists a number of different symptoms both for the acute phase (up to 100 days after radiation) as well as the late phase. The radiation effect on the lung has been investigated earlier in experimental animals (18-20).

BAL, in particular the measurement of phospholipids, has been widely used in investigating septic or polytraumatic shock-induced ARDS (21) and severe infant respiratory distress syndrome (IRDS) (22), as well as in determining effects of toxic drugs and dust to the lung (23-25). The importance of surfactant phospholipids has been reported (26-28). There are a

number of important indications that, in terms of the underlying pathogenic mechanisms, ARDS and IIP are somewhat similar to radiation-induced dysfunction (29). BAL gives insights into the regulatory and functional processes in the lungs before larger morphological changes occur. Changes in the surfactant phospholipid content are controlled by the P II cells, which can secrete regulatory mediator substances. As described (unpublished observations), significant short-term effects after irradiation (2 hr) are seen in the cellular and biochemical alveolar microenvironment. These findings could also be seen after total body irradiation of beagles (4.2 Gy ^{60}Co) (Quabeck and Rehn, personal communication). The cellular potency (phagocytic activity) is altered by irradiation in a time-dependent manner even after 10 Gy thoracic irradiation.

A rapid response of the surfactant phospholipid system has been described with significant changes in fractions of individual phospholipids, which shows extremely species-specific values for the mouse or rat (30). It also became clear that these fractions showed different temporal responses. These data may possibly extend previous measurements of surfactant phospholipid content. Thus, Rubin et al. (20) found no changes in single irradiated mouse lungs up to 13 Gy. A further subdivision may perhaps have shown such changes even at low doses. It must be pointed out here that the biological functions of individual components are only poorly understood (26-28). However, other areas of medicine have increasingly recognized the clinical importance of surfactant phospholipids. The substitution of surfactant phospholipids has already achieved marked successes in IRDS and ARDS (21,22). Alteration to the regulatory cycle of P II cell-surfactant phospholipid-macrophage may possibly be an important basis for radiation-induced interstitial pneumonitis and may be predictive of the syndrome. The high rate of pneumological complications of 30-60% in patients conditioned with high dose radio- and chemotherapy for bone marrow transplants (2,6) shows that a clarification of these pathogenic mechanisms achieves particularly urgent clinical significance. In this respect such BAL studies are already being carried out in Essen in bone marrow transplant and shock-lung patients.

The aim of the present investigations was to clarify mechanisms. It could be shown that there was an increase in the surfactant phospholipid content and in the number of granulocytes and lymphocytes before acute inflammation. The alveolar system responds with a marked reduction in the total surfactant phospholipid content and number of macrophages. The macrophages of the patient have apparently not been damaged in terms of viability and function; this could be definitely shown by the means of viable staining and functional testing of their phagocytic activity. The decrease in the total cell count is possibly a result of the decreased supply from the bone marrow, which has been damaged. These factors together then lead to a collapse of the entire surfactant-P II cells-alveolar macrophage system. Previous studies have shown that a chronic, irreversible process of fibrosis then occurs in this interaction of the altered alveolar microenvironment and the interstitium. Based on this case report, it could be shown that the prednisone treatment a) stimulated the production of surfactant phospholipid tremendously and b) by suppressing a probable pulmonary graft versus host response allowed sufficient macrophages to come in. As a result, the escalation of the acute inflammatory response was able to be cut off in the alveolar microenvironment.

Table 1. Total cell counts, macrophages, granulocytes, and lymphocytes in the bronchoalveolar lavage of a 43-year-old patient with idiopathic interstitial pneumonitis after bone marrow transplantation.

Days after transplant	Total cell count, cells/mL BAL	Macrophages, %	Lymphocytes, %	Granulocytes, %
107	820,000	14	43	43
109	4,070,000	17	48	35
145	800,000	48	27	25

BAL, bronchoalveolar lavage.

Table 2. Surfactant and sphingomyelin content in the bronchoalveolar lavage of a 43-year-old patient with idiopathic interstitial pneumonitis after bone marrow transplantation.

Days after transplant	Total phospholipids, $\mu\text{g/mL}$	Sphingomyelin, $\mu\text{g/mL}$
107	4,032	ND
109	7,595	165
145	45,954	37

ND, not detectable.

Table 3. Phagocytic activities of macrophages in the bronchoalveolar lavage of a 43-year-old patient with idiopathic interstitial pneumonitis after bone marrow transplantation.

Days after transplant	Macrophages, cells/dish	Phagocytizing, %	Nonphagocytizing, %
107	500,000	82	17
109	500,000	79	21
145	500,000	81	19

In conclusion, it could be demonstrated that complex events, e.g., those of inflammation, can be recognized as different parameters that are complementary to one another; even their exclusion, as demonstrated for this patient, can be important. In connection with the morphometric studies of lung fibrosis (9), a radiation-induced injury primarily toxic to the lungs can be ruled out.

REFERENCES

- Henderson, R. F. Use of bronchoalveolar lavage to detect lung damage. In: *Toxicology of the Lung* (D. E. Gardner, J. D. Crapo, and E. J. Massaro, Eds.), Raven Press, New York, 1988, pp. 239-267.
- Chan, K. C., Hylan, R. H., Hutcheon, M. A., Minden, M. D., Alexander, M. A., Kossakowska, A. E., Urbanski, S. J., Fyles, G. M., Fraser, I. M., Curtis, J. E., and Messner, H. A. Small-airways disease in recipients of allogeneic bone marrow transplants. *Medicine* 66(5): 327-340 (1987).
- Bamberg, M., Beelen, D. W., Mahmoud, H. K., Molls, M., and Schaefer, U. W. The incidence of interstitial pneumonitis: a comparison of total body irradiation schedules for allogeneic bone marrow transplantation. *Strahlenther. Onkol.* 162: 218-222 (1986).
- Krowka, M. J., Rosenow, E. C., and Hoagland, H. C. Pulmonary complications of bone marrow transplantation. *Chest* 87: 237-246 (1985).
- Weiner, R. S., Bortin, M. M., Gale, R. P., Gluckmann, E., Kay, H. E. M., Kolb, H. J., Hartz, A. J., and Rimm, A. A. Interstitial pneumonitis after bone marrow transplantation. Assessment of risk factors. *Ann. Intern. Med.* 104: 168-175 (1986).
- Wingard, J. R., Mellits, E. D., Sostrin, M. B., Yen-hung Chen, D., Burn, W. H., Santos, G. W., Vriesendorp, H. M., Beschoner, W. E., and Sara, R. Interstitial pneumonitis after allogeneic bone marrow transplantation. Nine-year experience at a single institution. *Medicine* 67(3): 175-186 (1988).
- Rehn, B., Bruch, J., Zou, T., and Hobusch, G. The flushability of rat alveolar macrophages under normal and activated conditions by bronchoalveolar lavage. *Environ. Health Perspect.* 97: 11-16 (1992).
- Davies, B. J., and Ornstein, L. High resolution enzyme localisation with a new diazo reagent, "hexazorium pararosaniline." *J. Histochem. Cytochem.* 7: 297-298 (1959).
- Kraus, R., Steinberg, F., Rehn, B., Bruch, J., and Streffer, C. Radiation induced changes in lung tissues and development of fibrosis revealed by quantitative morphometric methods. *J. Cancer Res. Clin. Oncol.* 117: 27-32 (1991).
- Gono, E., Rehn, B., Bruch, J., Pison, U., and Joos, M. R. Reaktion des Surfactants nach Staubbelaugung-Ubersicht und erste Ergebnisse. *Silikosebericht NW* 16: 237-244 (1987).
- Folch, J., Lees, M., and Stanley, G. H. S. A simple method for the isolation and purification of total lipids from animal tissue. *J. Biol. Chem.* 226: 497-509 (1957).
- Leskinen, R., Taskinen, E., Volin, E., Tukiainen, P., Ruutu, T., and Hayry, P. Use of bronchoalveolar cytology and determination of protein contents in pulmonary complications of bone marrow transplant recipients. *Bone Marrow Transplant.* 5: 241-245 (1990).
- Beelen, D. W., Quabeck, K., Graeven, U., Sayer, H. G., and Schaefer, U. W. Acute toxicity and first clinical results of intensive postinduction therapy using a modified busulfan and cyclophosphamide regimen with autologous bone marrow rescue in first remission of acute myeloid leukemia. *Blood* 74: 1507-1516 (1989).
- Holster, D. R. Lunge. In: *Strahlenpathologie der Zelle* (E. Scherer and H. S. Stender, Eds.), Georg Thieme, Stuttgart, 1963, pp. 265-277.
- Molls, M., and van Beuning, D. Strahlenbiologische Veränderungen der Lunge. In: *Handbuch der Medizinischen Radiologie* (F. Heuck and E. Scherer, Eds.), Springer-Verlag, Berlin, 1985, pp. 379-402.
- Hall, E. J., Marchese, M., Rubin, J., and Zaider, M. Low-dose rate irradiation. *Front. Radiat. Ther. Oncol.* 22: 19-29 (1988).
- Hamilton, C. R., Cummings, B. J., and Harwood, A. R. Radiotherapy of Kaposi's sarcoma. *Int. J. Radiat. Oncol. Biol. Phys.* 12(11): 1931-1935 (1986).
- Bublitz, G. Morphologische und biochemische Untersuchungen über das Verhalten des Bindegewebes bei der strahlenbedingten Lungenfibrose. Georg Thieme, Stuttgart, 1973.
- Phillips, Th. L., Wharam, M. D., and Margolis, L. W. Modification of radiation injury to normal tissue by chemotherapeutic agents. *Cancer* 35: 1678-1684 (1975).
- Rubin, P., Siemann, D. W., Shapiro, D. L., Finkelstein, J. N., and Penney, D. P. Surfactant release as an early measure of radiation pneumonitis. *Int. J. Radiat. Oncol. Biol. Phys.* 9: 1669-1673 (1983).
- Pison, U., Gono, E., Joka, Th., and Obertacke, U. Phospholipid lung profile in ARDS—evidence for surfactant abnormality. In: *First Vienna Shock Forum: Pathophysiological Role of Mediators and Mediator Inhibitors in Shock* (G. Schlag and H. Red, Eds.), Alan R. Liss, Inc., 1987, pp. 517-523.
- Collaborative European Multicenter Study Group. An international randomized clinical trial of surfactant replacement therapy in severe neonatal respiratory distress syndrome. *Pediatric* 82: 683-691 (1988).
- Bigby, T. D., Allen, D., Leslie, C. D., Henson, P. M., and Cherniack, R. M. Bleomycin-induced lung injury in the rabbit. *Am. Rev. Respir. Dis.* 132: 590-595 (1985).
- Lemaire, I. Characterization of the bronchoalveolar cellular response in experimental asbestosis. *Am. Rev. Respir. Dis.* 131: 144-149 (1985).
- Dethloff, L. A., Gladen, B. C., Gilmore, L. B., and Hook, G. E. R. Quantitation of cellular and extracellular constituents of the pulmonary lining in rats by using bronchoalveolar lavage. *Am. Rev. Respir. Dis.* 136: 899-907 (1987).
- Batenburg, J. J. Biosynthesis and secretion of pulmonary surfactant. In: *Pulmonary Surfactant* (B. Robertson, L. M. G. Van Golde, and J. J. Batenburg, Eds.), Elsevier Science Publishers, Amsterdam, 1984, pp. 237-270.
- Stratton, C. J. Morphology of surfactant producing cells and of the alveolar lining layer. In: *Pulmonary Surfactant* (B. Robertson, L. M. G. Van Golde, and J. J. Batenburg, Eds.), Elsevier Science Publishers, Amsterdam, 1984, pp. 68-118.
- Wright, J. R., and Clements, J. R. Metabolism and turnover of lung surfactant. *Am. Rev. Respir. Dis.* 136: 426-444 (1987).
- Wesseli, L. J., and Kimler, B. F. Alveolar macrophage proliferation in situ after thoracic irradiation rats. *Am. Rev. Respir. Dis.* 139: 221-225 (1989).
- Hobusch, G., Steinberg, F., Rehn, B., Gono, E., Bruch, J., and Streffer, C. Changes in bronchoalveolar lavage (BAL) after irradiation in Wistar rats. In: *Abstracts of the Seventh Annual Meeting of the European Society for Therapeutic Radiology and Oncology*, Den Haag, September 5-8, 1988, p. 107.